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�54 Oligonucleotides and determination system of HCV genotypes.

�57 Hepatitis C virus (HCV) oligonucleotides and a system to detect HCV and determine hepatitis C virus genotypes with polymerase chain reaction utilizing those oligonucleotides as primers.

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Background of the Invention

The present invention concerns hepatitis C virus (hereinafter called HCV) oligonucleotides and a system to determine HCV genotypes with polymerase chain reaction (PCR) utilizing those oligonucleotides as primers.

Viral hepatitis of which DNA and RNA of the causative viruses have been elucidated, and their diagnosis and even prevention in some cases have been established, are hepatitis A and hepatitis B. The general name NANB (non-A, non-B) hepatitis was given to the other forms of viral hepatitis.

Post-transfusion hepatitis was remarkably reduced after introduction of diagnostic systems for screening hepatitis B virus in transfusion bloods. However, there are still an estimated 280,000 annual cases of post-transfusion hepatitis caused by NANB hepatitis virus in Japan.

NANB hepatitis viruses were recently named C, D and E according to their types, and scientists started a world wide effort to conduct research for the causative viruses.

In 1988, Chiron Corp. claimed that it had succeeded in cloning RNA virus genome, which it termed hepatitis C virus (HCV), as the causative agent of NANB hepatitis and reported on its nucleotide sequence (British Patent 2,212,511 which is the equivalent of European Patent Application 0,318,216). HCV (c100-3) antibody detection systems based on the sequence are now being introduced for screening of transfusion bloods and for diagnosis of patients in Japan and in many other countries.

The detection systems for the c100-3 antibody have proven their partial association with NANB hepatitis; however, they capture only about 70% of carriers and chronic hepatitis patients, or they fail to detect the antibody in acute phase infection, thus leaving problems yet to be solved even after development of the c100-3 antibody by Chiron Corp.

The genome structure of HCV, or NANB hepatitis having relation to c100-3 antibody, has single stranded RNA which suggests some relationship to Flaviviruses and Pestiviruses. From the comparison of those genome structures, regions coding core protein (C), envelope protein (E) and non-structure protein (NS) were found in HCV. c100-3 antibody detected by Chiron's ELISA kit is thought to recognize a part of the border region of NS3/NS4.

The course of NANB hepatitis is troublesome and many cases of horizontal transmission are considered to become carriers and then to develop chronic hepatitis. In addition, most patients with chronic hepatitis develop liver cirrhosis, then hepatocellular carcinoma. It is therefore very imperative to isolate the virus itself and to develop effective diagnostic reagents enabling earlier diagnosis.

The presence of a number of NANB hepatitis which can not be diagnosed by Chiron's HCV (c100-3) antibody detection kits suggests the importance of detection of antibody to core protein instead of antibody to non-structural protein. From that. view point, the genome of the core region and the protein coded for by this region have been studied world wide.

From the two-by-two comparison of nucleotide sequences of various HCV isolates, homologies for the core region, which is rather conservative, were found to be less than 90% in some cases. In addition, more variability was found in the envelope region (which is thought to be important for development of vaccine); less than 60% homology was found between some isolates. The sequence diversity between strains reflects the features of HCV as RNA virus.

A rapid and simple method of classifying of HCV genomes into groups, therefore, would be very useful for diagnosis, therapy and prevention of NANB hepatitis.

Determination of HCV genotypes could be useful in the following medical and social applications:

(1) identification of infectious sources in vertical (mother-to-baby) transmission and horizontal transmission;

(2) clarification of genotype-specific features in the course, condition and prediction of recuperation of NANB liver diseases and making a guide of therapy therefore:

(3) increasing the detection sensitivity of HCV by nucleic acid detection system or antibody detection system based on the study of diversity in nucleotide or amino acid sequences among HCV isolates of distinct genotypes;

(4) production of type-specific immunoglobulin (IgG) and vaccine based on the study of type-specific conservative regions in the envelope gene of various genotypes; and

(5) rapid and effective prevention by selecting an appropriate IgG or vaccine for each genotype.

The inventors have obtained various HCV-RNAs from sera of humans and chimpanzees, and cloned cDNAs of HCV genomes in non-coding regions and coding regions for core and envelope proteins, followed by determination of nucleotide sequences (Japanese Patent Applications 196175/91, 287402/91 and 360441/91), and sought to find a method to determine HCV genotypes and oligonucleotide primers to be used in said method, which would be useful in diagnosis, therapy, prevention, identification of infectious source, and epidemiological study on HCV. As a result, variation of nucleotide sequence characteristics in some groups

in the core region, which was thought to be rather conservative, was found, and much more variation was found in th nvelope r gion.

Summary f th Inv ntion

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It has be n f und as a result f study of a great numb r of nucleotide sequences of HCV isolates that HCVs could be divided into four genotypes through the nucleotide sequence of the putative C gene and based on the homology of nucleotides. Based on this finding, universal oligonucleotides and type-specific oligonucleotides were identified and synthesized.

10   In accordance with one aspect of the invention, there is provided universal oligonucleotides #104, #186, and #256 and type-specific oligonucleotides #132, #133, #134, #135 and #296.

A further feature of the invention resides in a method of determining HCV genotypes by polymerase chain reaction (PCR) amplification of cDNA using one or more of said oligonucleotide as primers. The method of determining HCV genotypes could be carried out by measuring lengths of amplification products obtained by

15   PCR described above.

Thus, in accordance with a further aspect of the invention, there is provided a method of determining HCV genotypes. The method involves two stage PCR, wherein the second stage PCR comprises utilizing one or more of oligonucleotides #132, #133, #134, #135 and #296 as antisense primer and utilizing one or more of oligonucleotides #104, #23 and #256 as sense primer. The method further involves first stage PCR utilizing

20   one or both of oligonucleotides #23 and #256 as sense primer and #186 as antisense primer.

The method to determine genotypes can be used in a method of predicting response of a patient with type C hepatitis to drug therapy.

Brief Description of the Drawings

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Figure 1 is a table which shows the results of classification of genotypes and nucleotide sequences corresponding to primers on 39 samples.

Figure 2 is a diagram which shows the structure of the coding region of NANB hepatitis virus genome and PCR products using primers of the invention.

30   Figure 3 is a photo which shows electrophoresis pattern of genotypes 1 to IV. Indicated on the left lane are migration positions of molecular size markers (øX174-RF DNA HaeIII digest; Pharmacia LKB).

Figure 4 is a photo which shows electrophoresis pattern of double infection cases. Migration positions of PCR products of the four different HCV types are indicated on the right.

Figure 5 is a photo which shows electrophoresis pattern of identification of infectious sources. Migration

35   positions of PCR products of the four different HCV types are indicated on the right.

Figure 6 is a table which shows nucleotide sequence of HCV in #256 and #296 region in 39 samples.

Figure 7 is a photo which shows electrophoresis pattern of 6 HCV samples. Indicated on the left lane are migration positions of molecular size markers (øX174-RF DNA HaeIII digest; Pharmacia LKB).

Figure 8 is a photo which shows electrophoresis pattern of four HCV samples. Molecular markers in the

40   left lane are 100bp-ladder (Gibco, BRL).

In Figures 3 - 5, 7 and 8, I, II, III and IV show genotypes I, II, III and IV of HCV. 1, 2, 3, 4, 5 and 6 are lanes in electrophoresis pattern.

Detailed Description of the Invention

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In accordance with the present invention, there is provided a group of new oligonucleotides; in particular, new oligonucleotide primers #132, #133, #134, #135, #104, #23, #256, #186 and #296, which were obtained by chemical synthesis. These primers have the sequences shown in the attached sequence listing.

The novel oligonucleotides of the invention were determined as follows:

50   In a first step, HCV-cDNAs spanning the putative C gene were isolated from 39 serum or plasma samples from humans or chimpanzees, and nucleotide sequences of the cDNAs were determined as shown herein.

Four regions consisting of 20 successive nucleotides in common with all the cDNAs were selected at nt 148-167, nt 126-145, nt 139-158 and nt 391-410.

In the next step, regions w re sel cted which consist d of 20 successive nucl otid s which had high hom-

55   ology (basically containing less than 3 substituti ns) in some cDNAs but showed low homology (basically containing more than 2 substitutions, and many substitutions observed in 5' terminus) in other cDNAs at nt 185-204, nt 272-291, nt 302-321 and nt 251-270.

It was confirmed by the present invention that all the HCV-cDNAs could b classifi d into four groups when

classified by the nucleotide sequence of five regions nt 185-204, nt 272-291, nt 302-321, nt 251-270 and nt 177-196 without an v rlap.

Four nucl otid s corr sponding to r gions having cons rvativ sequ nces above were synth siz d with Cyclone Plus DNA Synthesizer as #104 (nt 148-167), #23 (nt 126-145), #256 (nt 139-158) and #186 (nt 391-
5   410). In the same manner, five nucleotid s having complementary sequences to type-sp cific regions of HCV g n mes abov were synthesized as #132 (nt 185-204), #133 (nt 272-291), #134 (nt 302-321), #135 (nt 251-270) and #296 (nt 177-196).

The method of classification of HCV genotypes by amplifying cDNA using oligonucleotide primers #132, #133, #134, #135 and #296 having a complementary sequence to each region of HCV genomes as antisense
10   primers, was completed.

Oligonucleotide primers #23 (nt 148-167), #104 (nt 126-145), #256 (nt 139-158) and #186 (nt 391-410) are well-conserved among different genotypes, and can be utilized as universal sense primers or antisense primers.

A preferred way to carry out the method of determining HCV genotypes is as follows: C-region cDNA is
15   obtained by reverse transcription with primer #186, and the cDNA is amplified using oligonucleotide primer #23 or #256 as a sense primer and #186 as an antisense primer in the first stage PCR followed by amplification using #104 as a sense primer and #132, #133, #134 and #135 as antisense primers in the second stage PCR.

Since PCR products can be separated by electrophoresis and observed under u.v. light, the genotypes can be identified according to the length of the amplification products of the second stage PCR.
20   When previously isolated HCV genomes were classified by the method of the invention, they were divided into each one of four groups of type I, II, III and IV.

Names of genotypes were defined as follows; Type I: HCV group which can be amplified using antisense primer #132 and/or #296, and in the same manner, type II: #133, type III: #134, and type IV: #135.

HCV clones HC-J1, HC-J4, HC-J5, HC-J6, HC-J7 and HC-J8 , which were isolated by the inventors and
25   disclosed in the above-identified Japanese patent applications, were classified into type I, II, III, III, IV and IV respectively using the method of determining HCV genotypes.

As shown in the Examples, 39 HCVcDNA clones obtained from serum or plasma of humans or chimpanzees were classified into each one of four genotypes I-IV. The homology of the nucleotide sequence of 39 samples were examined at the putative C gene nt 146-486. As a result, the homology was calculated as 95.1-97.5%
30   among isolates classified into a genotype, while it was calculated as 77.9-91.0% among isolates classified into different genotypes. The results show that the method of the invention can identify HCV genotypes efficiently. (see Table 1; values represent mean ± S.D. percentage identity)

Table 1. Degree of sequence identity in a part of the putative C gene within and
35   among four different types of HCV

Number of isolates	Type	I	II	III	IV
4	I	97.4±0.3			
20	II	91.0±1.0	95.3±1.2		
10	III	79.5±0.8	79.4±1.1	95.1±1.0	
5	IV	79.1±0.8	77.9±0.7	85.5±0.8	96.1±0.6

45   In the specification, "nt" was numbered from the start of the single open reading frame, and "nc-nt" was numbered from putative 5′ terminus of 5′ non-coding region of the HCV genome.

Genotypes of Japanese sera from patients with HCV infection were examined by the method of the invention. As a result, as much as 82% were classified into type II, then 10%, 4% and 4% were respectively classified into type III, I and IV, among asymptomatic (healthy) carriers. In contrast, 60% were classified into type
50   II, and 23% into type III among patients with chronic NANB liver disease. (Table 2) This result suggested that type III has higher possibility to cause an outbreak of chronic hepatitis than type II.

As described above, the method of the invention was shown to be useful in prevention and prediction of prognosis of HCV infection and type C chronic liver diseases.

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Table 2. Prevalence of four HCV typ s in blood donors and patients with HCV infection						
D n r or patient	Sampl number	Genotype				
		I	II	III	IV	Mixed
Blood donors	159	6 (4%)	131 (82%)	16 (10%)	6 (4%)	0
Acute hepatatis	12	0 (0%)	9 (75%)	3 (25%)	0 (0%)	0
Chronic hepata-tis	27	0 (0%)	16 (59%)	7 (26%)	3 (11%)	1 (I/II)
Liver cirrhosis	24	2 (8%)	15 (63%)	5 (21%)	2 (8%)	0
Hepatocellular carcinoma	17	2 (11%)	8 (47%)	3 (18%)	3 (18%)	1 (II/III)
Hemophiliac	11	3 (27%)	3 (27%)	2 (18%)	1 (9%)	2 (I/II,I/III)

The method of the invention enables identification of HCV double infection and tracing of the source of infection.

In addition, as described in the Examples, significance was found in responses to interferon (hereinafter IFN) between types II and III among patients with chronic hepatitis C. This finding made it clear that the method to determine HCV genotypes of the invention is very useful in predicting the efficiency of drug therapy against hepatitis C.

The invention provides a method to definitely determine HCV genotypes, and it will be useful in many applications e.g. development of HCV IgG and vaccine, and establishment of the manner to administer them.

The invention provides a method to predict response of a patient with type C hepatitis to drug therapy.

The invention also provides oligonucleotides which can be utilized as sense primers or antisense primers in the method of determining HCV genotypes.

Examples of applications of this invention are shown below, however, the invention shall in no way be limited to those examples.

Examples

Example 1

Oligonucleotide #132, #133, #134, #135, #104, #23, #256, #186 and #296 were determined in the following way.

(1) Isolation of RNA

RNA of serum or plasma samples from Japanese blood donors testing positive for HCV antibody (c100-3 antibody; by Ortho HCV Ab ELISA, Ortho Diagnostic System, Tokyo), and that from the chimpanzee challenged with NANB hepatitis for infectivity and negative for HCV antibody, were isolated in the following method:

150 ml of each plasma sample was added to 150 ml of Tris chloride buffer (50 mM, pH 8.0, containing 150 ml NaCl, 10 mM EDTA) and centrifuged at 90 x 10^3 rpm for 15 minutes. The precipitate was suspended in Tris-chloride buffer (50 mM, pH 8.0, containing 200 mM NaCl, 10 mM EDTA, 2% (w/v) sodium dodecyl sulfate (SDS) and proteinase K 1 mg/ml), and incubated at 60° for 1 hour. Then the nucleic acids were extracted by phenol/chloroform and precipitated by ethanol to obtain RNAs.

(2) Detection of 5' non-coding region RNA and core region RNA by Polymerase Chain Reaction (PCR)

The isolated RNA were heated at 70° for 1 minute; using this as a template, cDNA was synthesized using oligonucleotide primer #36 (5' - A A C A C T A C T C G G C T A G C A G T - 3', nc-nt 246-265: antisense) and reverse transcriptase (Superscript, BRL, U.S.A.), and was provided to PCR as follows.

cDNA was amplifi d according to Saiki's method (Science (1988), volume 239, pages 487-491) using Gene Amp DNA Amplification Reagent (Perkin-Elmer Cetus) on a DNA Thermal Cycl r (Perkin-Elmer Cetus). PCR was performed in two stages using 5' non-coding specific prim r #32A (5' - C T G T G A G G A A C T A C T G

T C T T - 3', nc-nt 45-64: sense) and #36 in the first stage; and #33 (5' - T T C A C G C A G A A A G C G T C
T A G - 3', nc-nt 63-82: sens) and #48 (5' - G T T G A T C C A A G A A A G G A C C C - 3', nc-nt 188-207:
antis ns) locating insid f #32A and #36, in th s cond stag .

28 samples obtain d from sera f Japan s blood donors, 10 from patients with NANB liv r dis ase, and
one from chimpanzee observed to b infected with NANB h patitis, all testing positiv for 5' non-coding r gion
RNA by said PCR in two stages, w r provid d to amplification of the C gene.

cDNA was synthesized for each sample using oligonucleotide #122 (5' - A G G T T C C C T G T T G C A
T A A T T - 3', nt 487-506: antisense) as a primer, followed by generation of a product of 381bp representing
nt 126-506 corresponding to two third of C gene by PCR using #23 and #122.

PCR was carried out by the following reaction cycle: denaturation at 94°C for 1 minute, annealing of primers
at 55°C for 1.5 minutes, and extension at 72°C for 2 minutes. The products were cloned into the HincII site of
M13mp19 phage vector. cDNA clones were sequenced in both directions by dideoxynucleotide chain termin-
ation method by the use of Sequenase version 2.0 (United States Biochemical, U.S.A.).

(3) Determination of universal primers and type-specific primers

In the nucleotide sequence of the obtained clones, some regions consisting of 20 successive nucleotides
having conservative sequence (having substitution of less than 3 based in 20 bases) were selected. Nucleotide
sequence in common with all the cDNAs were observed in regions nt 148-167, nt 126-145, nt 139-158 and nt
391-410. On the other hand, regions nt 177-196, nt 185-204, nt 272-291, nt 302-321 and nt 251-270 were rec-
ognized conservative in some isolates but not in common with others, which were thought to be genotype spe-
cific. (Figure 1, Figure 6).

Based on consensus sequence of each region, oligonucleotides #104 (sequence list 5, nt 148-167: sense),
#23 (sequence list 6, nt 126-145: sense), #256 (sequence list 7, nt 139-158: sense) and #186 (sequence list
8, nt 391-410: antisense) were specified as universal primers.

In the same manner, oligonucleotides #132 (sequence list 1, nt 185-204: antisense), #133 (sequence list
2, nt 272-291: antisense), #134 (sequence list 3, nt 302-321: antisense), #135 (sequence list 4, nt 251-270:
antisense) and #296 (sequence list 9, nt 177-196: antisense) were specified as type-specific primers.

Said oligonucleotides #104, #23, #256, #186, #132, #133, #134, #135 and #296 were synthesized with
Cyclone Plus DNA Synthesizer (MilliGen/Biosearch, Division of Millipore) in the usual way.

Example 2-Genotype determination system of the invention

(1) Typing of HCV-RNA positive samples

Genotypes were determined using primers obtained in Example 1.

cDNAs were obtained using primer #186 from 39 sera used in Example 1. The reaction cycle was repeated
35 times with denaturation at 94°C for 1 minute, primer annealing at 55°C for 1.5 minutes, and primer extension
at 72°C for 2 minutes.

In the first stage PCR, 272 nucleotides of nt 139-410 were amplified with #256 and #186 for 35 cycles
with a reaction cycle as described above.

A 1/50 amount of the products was subjected to second PCR for 30 cycles using a universal primer #104
and a mixture of equivalent amount of four type-specific primers, #132, #133, #134 and #135, with denaturation
at 94°C for 1 minute, primer annealing at 60°C for 1 minute, and primer extension at 72°C for 1.5 minutes. The
products of the second PCR were subjected to electrophoresis on a composite agarose gel made from 1.5%
NuSieve and 1.5% SeaKem (FMC BioProducts, U.S.A.), stained with ethidium bromide, and observed under
u.v. light.

The products were identified by molecular sizes. That is, since a product amplified with #132 consists of
57bp, and that with #133, #134 and #135 consist respectively of 144bp, 174bp and 123bp, the position of a
product shows its molecular sizes, and the primer used for amplification therefor. (Figure 3).

Genotypes of 39 sera were completely identified as same result in Example I-(3) (Figure 1), showing ex-
cellent specificity of type-specific primers of the invention.

Genotypes I, II, III and IV show types amplified with primer #132, #133, #134 and #135.

The same procedure with primer #23 in place of #256 in the first stage PCR gave the same result as (1)
above.

(2) The same procedure with primer #23 in plac of #104 in the s cond stag PCR gave sam r sult as
(1) abov .

Th sam procedure with primer #256 in place of #104 in the second stag PCR gave the sam result as

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(1) above.

(3) Variability of nucleotide sequ nce in nt 146-486 of C g n were xamined on 39 s ra classified into g notypes in (1) above. The clon s classified into a g n typ , which w r amplified by on f the mix d antisense prim rs, showed homology as high as 95.1-97.5% while thos classifi d into the other gen types sh w d homology as low as 77.9-91.0%. (Tabl 1)

cDNAs of HC-J1, HC-J4, HC-J5, HC-J6 and HC-J7 or HCV isolates disclosed in previous Japanese applications were subjected to the method of determining genotypes using #132, #133, #134 and #135 as mixed antisense primers. As a result, HC-J1 was classified into type I, and HC-J4, HC-J5, HC-J6 and HC-J7 were respectively classified into types II, III, III and IV.

Example 3

Test sera from 96 patients, diagnosed histopathologically by biopsy as chronic hepatitis C and testing positive for HCV-RNA by PCR amplifying 5' non-coding gene, were subjected to classification of HCV genotypes in the same manner described in Example 2-(1). There was found 69 as type II (72%), 21 as type III (22%) and 6 as type IV (6%), where none was found as type I.

Drug therapy were carried out for said 96 patients with interferon α. Recombinant interferon-α2a (Roferon A, Nippon Roche, Tokyo) was administered subcutaneously 6MU/day for seven successive days, followed by 3MU/day for three days a week for 23 successive weeks.

Just after the 24-week IFN treatment, HCV-RNA was not found in 14 patients (20%) of type II, 14 (67%) of type III and 2 (33%) of type IV, from the result of PCR amplifying 5' non-coding gene. The HCV-RNA clearance rate of type II was found to be significantly higher than that of gene type III (p<0.001, Wilcoxon test). Moreover, type III showed very low HCV-RNA level after IFN treatment compared with types II and IV as shown in Table 3. For the level of HCV-RNA in Table 3, values of 1, 2, 3, etc. represent 10^0, 10^1, 10^2, etc. CIU (Chimpanzee Infectious Unit)/ml.

Table 3. HCV genotypes and response of chronic hepatitis C patients to interferon therapy.				
Genotypes	Patients number	HCV-RNA quantity		HCV-RNA eliminated cases
		before IFN	after IFN	
II	69	5.2±1.2	2.7±2.0	14 (20.3%)
III	21	4.7±1.1	0.8±1.3	14 (66.7%)
IV	6	4.8±1.6	3.3±2.8	2(33.3%)
Total	96	5.1±1.2	2.3±2.1	30 (31.3%)

As a result, some difference in response to interferon among genotypes was recognized among genotypes (which were classified by the method of determining genotypes). It became clear, therefore, that the invention is very useful in predicting the efficiency of drug therapy against hepatitis C. A study similar to that above can be conducted using other drugs or modalities of treatment. Differences in response of the four types of HCV to the treatment can thus be determined. Such data can then be used to predict the response to treatment of a patient infected with a strain of HCV. After the genotype of the patient's strain has been identified, the data from such a study will indicate the response to treatment of that strain which belongs to a particular type. For example, table 3 indicates that a patient infected with HCV type III would respond better to treatment with IFN than a patient infected with HCV type II.

Example 4

(1) HCV genotyping of Japanese cases

For the epidemiological study on HCV genotypes in Japan, samples were obtained from 159 blood donors, 80 patients with NANB liver disease and 11 hemophiliacs. HCV-RNAs were isolated from those sera, followed by cDNA synthesis and PCR to determine g notypes according to th procedur described in Example 2.

As the result, more than 80% of blood donors were typed as II as shown in Table 2. On the other hand, 48 of 80 patients with NANB liver diseases (60%) were typed as II, a significant difference (p<0.01) was recognized. Among those with hepatocellular carcinoma, only 48% were typed as II, while I (11%), III (18%), IV

(18%) were found more often than among blood donors.

Wh n blood d n rs and patients with NANB liv r dis ase were compared on typ III, 10% (16/159) w re found in th former group, and 23% (18/80) in th latter gr up, a significant difference (p<0.01) was recognized. Significance was evaluated by Chi-squared analysis.

Five samples were typed as I and 4 as II among hemophiliacs, including cases of suspected double inf c-tion. The results suggested that some coagulation factor concentrates imported from the United States in the distant past, which had not been heat-inactivated, were possibly contaminated with HCV.

From the above, the method of the invention provides easy classification of HCV genotypes. In addition, the existence among genotypes of differences in the clinical course of hepatitis after infection with HCV sug-gests the usefulness of the method in the therapy and protection of NANB liver disease. As shown above, the results suggest that type III has higher possibility to cause an outbreak of chronic hepatitis than type II.

(2) Identification of double infection

Four cases of double infection were observed as shown in the electrophoresis pattern in Figure 4. Two hemophiliacs (types I and II in lane 3; types I and III in lane 4) described in Example 3-(1), a patient with chronic hepatitis (types I and II in lane 1) and a patient with hepatoma (types II and III in lane 2) were included.

Double infection with HCV of different types in a case of a hemophiliac (lane 3) was confirmed by cloning of some cDNAs and sequence analysis within 341bp in the C gene (nt 146-486); an identity of more than 99% was observed between isolates of clones assumed to be the same type, while only 88.8% between those as-sumed to be distinct types. In the same manner, cDNAs obtained from another hemophiliac (lane 4), a patient with chronic hepatitis and that with hepatocellular carcinoma were also classified into two types, where iden-tities of 79.4%, 90.1% and 80.3% were respectively observed in distinct types. Those identities can be con-firmed to show distinct types when compared with results in Example 2-(3)(Table 1).

Compared with conventional techniques to study genotypes, that is to isolate some clones from a sample, in order to determine those nucleotide sequences and then to calculate identities between sequences, the method of the invention provides an expeditious method of detection of double infection by HCV of distinct types.

(3) Identification of infectious source in vertical and horizontal transmission

Test sera were obtained from 2 cases suspected of vertical (perinatal) transmission, and from a case of suspected horizontal transmission by needle stick exposure, and then subjected to determination of genotypes.

Figure 5 shows the result of electrophoresis of PCR products following cDNA synthesis and PCR of typing according to the method described in Example 2. In Figure 5, lane 1-4 were from two mother-to-baby pairs of perinatal transmission. Lane 1 and 2 were PCR products from a mother and baby of a family where genotypes of mother and baby were identified as type II; lanes 3 and 4 were from those of another family where genotypes of the mother and baby were identified as type III. Lane 5 and 6 were from a case of accidental HCV infection, where two genotypes from both donor (lane 5) and recipient (lane 6) were identical (type IV).

As the result, it became clear that the method of the invention is useful in tracing infectious sources of NANB hepatitis.

Example 5

HCV genomes HC-J2, HC-J3, (Japan J. Exp. Med. 60: 167-177, 1990) and HC-J8 (Japanese Patent Ap-plication No. 360441/91), which the inventors isolated and reported previously, were subjected to the deter-mination of genotypes.

In the first stage PCR, #256 was used as a sense primer, and #186 as an antisense primer. In the second stage PCR, #104 was used as a sense primer, and mixed primer of #132, #133, #134 and #135 was used as antisense primer according to the method described in Example 2-(1). HC-J1, HC-J4 and HC-J6 described above were subjected to the test for comparison.

Figure 7 shows the result of electrophoresis, where lanes 1-6 shows respectively HC-J1, HC-J2, HC-J3, HC-J4, HC-J6 and HC-J8. As the result, HC-J2 and HC-J3 were typed as II (the same as HC-J4), while HC-J8 was typed as IV.

In Examples above, primers were used selecting T for Y and G for R in #132; G for R, T for W and C for Y in #133; G for R in position five and A for R in position 16 in #134; A for R in positions one and 19, G for R in position 13 and C for Y in #135. With r gard to universal primers, they wer used selecting G for R in positions three, six, and 18, A for R in position seven and C for S in #104; A for M, T for N, G for R in position 15 and A

for R in position 16 in #256; T for Y and G for R in #186.

The method of inv ntion using primers s l cting the other possible nucleotides gav same results as de-scrib d above.

5 Exampl 6

Test sera No. 1, 2, 3 and 4, which were respectively typed as I, II, III and IV by the method described in Example 2-(1), were subjected to PCR for typing using equivalent mixture of primers #296, #133, #134 and #135 as antisense primer. cDNA priming and two stage PCR were carried out by the method described in Ex-

10 ample 2-(1) except primer #296 was used for #132. PCR products were observed under u.v. light (after elec-trophoresis in composite agarose gel of 2% NuSieve and 2% SeaKem and stained with ethidium bromide). (Figure 8)

As a result, the product generated by #296 (49bp) was observed for sample No. 1 (lane 1), and products generated by #133, #134 and #135 were observed for sample No. 2-4. It became clear that the method of the

15 invention using #296 instead of #132 gives the same result in determination of HCV genotypes.

The invention provides an expeditious method to classify HCV genomes into four types (genotypes).

The invention has many effects and applications, such as development of HC IgG and vaccine with high specificity and establishment of manner to administer them.

Moreover, the invention provides a method to identify HCV double infection and to track down the infec-

20 tious source of HCV, and provides an excellent method in the study of HCV and in diagnosis, prevention and therapy of hepatitis C.

Further variations and modifications of the invention will become apparent to those skilled in the art from the foregoing and are intended to be encompassed by the claims appended hereto.

Japanese Patent Application No. 307296/91 filed on September 9, 1991; 93960/92 filed on February 28,

25 1992; and a Japanese Patent Application filed on September 2, 1992, are all relied on and incorporated by reference; Hiroaki Okamoto and Tetsuo Nakamura are the inventors of all three Japanese applications.

Copending U.S. Patent Application Serial No. 07/712,875 filed on June 11, 1991 and 07/873,126 filed on April 24, 1992, are incorporated by reference in their entirety.

Okamoto et al., "Typing hepatitis C virus by polymerase chain reaction with type-specific primers: appli-

30 cation to clinical surveys and tracing infectious sources", Journal of General Virology (1992), volume 73, pages 673-679, is incorporated by reference in its entirety.

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Sequence list:

SEQ ID LIST 1 (#132)
(A)LENGTH: 20 base pairs
(B)TYPE: nucleic acid
(C)STRANDEDNESS: single
(D)TOPOLOGY: linear
(E)MOLECULE TYPE: cDNA to genomic RNA

SEQUENCE DESCRIPTION OF SEQ ID LIST 1

YRCCTTGGGG ATAGGCTGAC 20
(Y = T/C;   R = G/A)

SEQ ID LIST 2 (#133)
(A)LENGTH: 20 base pairs
(B)TYPE: nucleic acid
(C)STRANDEDNESS: single
(D)TOPOLOGY: linear
(E)MOLECULE TYPE: cDNA to genomic RNA

SEQUENCE DESCRIPTION OF SEQ ID LIST 2

GARCCAWCCT GCCCAYCCYA 20
(R = G/A;   W = T/A;   Y = C/T)

SEQ ID LIST 3 (#134)
(A)LENGTH: 20 base pairs
(B)TYPE: nucleic acid
(C)STRANDEDNESS: single
(D)TOPOLOGY: linear
(E)MOLECULE TYPE: cDNA to genomic RNA

SEQUENCE DESCRIPTION OF SEQ ID LIST 3

CCAARAGGGA CGGGARCCTC 20
(R = G/A)

SEQ ID LIST 4 (#135)
(A)LENGTH: 20 base pairs
(B)TYPE: nucleic acid
(C)STRANDEDNESS: single
(D)TOPOLOGY: linear
(E)MOLECULE TYPE: cDNA to genomic RNA

SEQUENCE DESCRIPTION OF SEQ ID LIST 4

RCCYTCGTTT CCRTACAGRG 20
(R = G/A;   Y = C/T)

SEQ ID LIST 5 (#104)
(A)LENGTH: 20 base pairs
(B)TYPE: nucleic acid
(C)STRANDEDNESS: single
(D)TOPOLOGY: linear
(E)MOLECULE TYPE: cDNA to genomic RNA

SEQUENCE DESCRIPTION OF SEQ ID LIST 5

AGRAARRCTT CSGAGCGRTC 20
(R = G/A;   S = C/G)

SEQ ID LIST 6 (#23)
(A)LENGTH: 20 base pairs
(B)TYPE: nucleic acid
(C)STRANDEDNESS: single
(D)TOPOLOGY: linear
(E)MOLECULE TYPE: cDNA to genomic RNA

SEQUENCE DESCRIPTION OF SEQ ID LIST 6

TAGATTGGGT GTGCGCGCGA 20

SEQ ID LIST 7 (#256)
(A)LENGTH: 20 base pairs
(B)TYPE: nucleic acid
(C)STRANDEDNESS: single
(D)TOPOLOGY: linear
(E)MOLECULE TYPE: cDNA to genomic RNA

SEQUENCE DESCRIPTION OF SEQ ID LIST 7

CGCGCGMCNA GGAARRCTTC 20
(M = A/C;   N = A/T/C/G;   R = A/G)

SEQ ID LIST 8 (#186)
(A)LENGTH: 20 base pairs
(B)TYPE: nucleic acid
(C)STRANDEDNESS: single
(D)TOPOLOGY: linear
(E)MOLECULE TYPE: cDNA to genomic RNA

SEQUENCE DESCRIPTION OF SEQ ID LIST 8

AYGTACCCCA YGAGRTCGGC 20
(Y = T/C;   R = G/A)

```
SEQ ID LIST 9 (#296)
(A)LENGTH: 20 base pairs
(B)TYPE: nucleic acid
(C)STRANDEDNESS: single
(D)TOPOLOGY: linear
(E)MOLECULE TYPE: cDNA to genomic RNA

SEQUENCE DESCRIPTION OF SEQ ID LIST 9

GGATAGGCTG ACGTCTACCT 20
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Claims

1. An oligonucleotide corresponding to the genome of hepatitis C comprising one or more of the nucleotide sequences of sequence lists 1-5 and 7-9 and the complements thereof.

2. An oligonucleotide as claimed in claim 1 wherein in the sequence of sequence list 1, Y is T and R is G.

3. An oligonucleotide as claimed in claim 1 wherein in the sequence of sequence list 2, R is G, W is T and Y is C.

4. An oligonucleotide as claimed in claim 1 wherein in the sequence of sequence list 3, the first occurrence of R is G and the second occurrence of R is A.

5. An oligonucleotide as claimed in claim 1 wherein in the sequence of sequence list 4, Y is C, the first occurrence of R is A, the second occurrence of R is G and the third occurrence of R is A.

6. An oligonucleotide as claimed in claim 1 wherein in the sequence of sequence list 5, S is C; the first, second and fourth occurrences of R is G, and the third occurrence of R is A.

7. An oligonucleotide as claimed in claim 1 wherein in the sequence of sequence list 7, M is A, N is T, and the first occurrence of R is G and the second occurrence of R is A.

8. An oligonucleotide as claimed in claim 1 wherein in the sequence of sequence list 8, Y is T and R is G.

9. A method of detecting HCV by nucleotide binding or hybridisation wherein an oligonucleotide comprising one or more of the nucleotide sequences of sequence lists 1-9 and the complements thereof is employed.

10. A method of determining HCV genotypes, said method comprising two stage PCR, wherein the second stage PCR comprises utilizing one or more of oligonucleotides #132, #133, #134, #135 and #296 as antisense primer and utilizing one or more of oligonucleotides #104, #23 and #256 as sense primer.

11. The method according to claim 10, wherein first stage PCR comprises utilizing one or both of oligonucleotides #23 and #256 as sense primer and #186 as antisense primer.

12. The method according to claim 10 or claim 11, further comprising measuring the molecular length of the resulting amplified cDNA.

13. A method to predict the response to drug therapy of a patient infected with a strain of HCV, said method comprising determining the sensitivity of HCV types I, II, III and IV to said drug therapy, determining the HCV genotype of said strain of HCV by the method according to claim 10, and comparing said HCV genotype of said strain with said sensitivity of HCV types I, II, III and IV to said drug therapy.

14. A kit for detecting the presence f HCV and/or determining the HCV genotype comprising at least one oligonucleotide comprising n or more of the nucleotide sequences of sequence lists 1-9 and th c m-plem nts there f.

Fig. 1

NUCLEOTIDE SEQUENCES AND POSITIONS OF UNIVERSAL AND TYPE-SPECIFIC PRIMERS

Fig. 2

(A) 1st PCR WITH UNIVERSAL PRIMERS

285 bp (nt 126–410)

(B) 2nd PCR WITH TYPE-SPECIFIC PRIMERS

TYPE I   57 bp (nt 148–204)

TYPE II   144 bp (nt 148–291)

TYPE III 174 bp (nt 148–321)

TYPE IV   123 bp (nt 148–270)

Fig. 3

Fig. 4

Fig. 5

Fig. 6

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TYPE  CLONE
        No.           #256 (S)                          #296 (AS)
              139                158           177                     196
              CGCGCGACTAGGAAGACTTC            AGGTAGACGTCAGCCTATCC
  I      1  ----------G---------              --------------------
         2  ----------G---------              --------------------
         3  ----------G---------              --------------------
         4  ----------G---------              --------------------

 II      5  --------------------              T—A—G—A—A——————
         6  ----------------G---              T—A—G—A—A——————
         7  --------------------              T—A—G—A—A——————
         8  --------------------              T—A—G—A—A——————
         9  --------------------              T—C—G—A—A——————
        10  --------------------              T—A—G—A—A——————
        11  --------------------              T—A—G—A—A——————
        12  --------------------              T—A—G—A—-A——————
        13  --------------------              T—A—G—A—A——————
        14  --------------------              T—G—G—A—A——————T—
        15  --------------------              T—A—G—A—A——————
        16  --------------------              T—A—G—A—A——————
        17  --------------------              T—A—G—A—A——————
        18  --------------------              T—G—G—A—A——————
        19  --------------------              T—A—G—A—A——————
        20  --------------------              T—A—G—A—A——————
        21  --------------------              T—A—G—A—A——————
        22  --------------------              T—A—G—A—A——————
        23  --------------------              T—A—G—A—A——————
        24  --------------------              T—A—G————A——————

III     25  ----------C-C-------              T—G—G—C——————C——————
        26  ----------A---------              T—A—G—C——————C——————
        27  ----------A---------              T—A—G—C——————C——————
        28  ----------A---------              C—G—G—C——————C——————
        29  ----------G---------              T—G—G—C——————C——————
        30  ----------A-------A--             T—G—G—C——————C——————
        31  ----------A---------              T—G—G—C——————C——————
        32  ----------A---------              T—G—G—C——————C——————
        33  ----------A---------              T—G—G—C——————C——————
        34  ----------A---------              T—AG—G—C——————C——————

 IV     35  ----------A---------              T—G——————C——————C———
        36  ----------G---------              T—G——————C——————C———
        37  ----------A---------              T—AC——————C——————C——————
        38  ----------A---------              T—G——————C——————C——————
        39  ----------A---------              T—G—G—C——————C——————
```
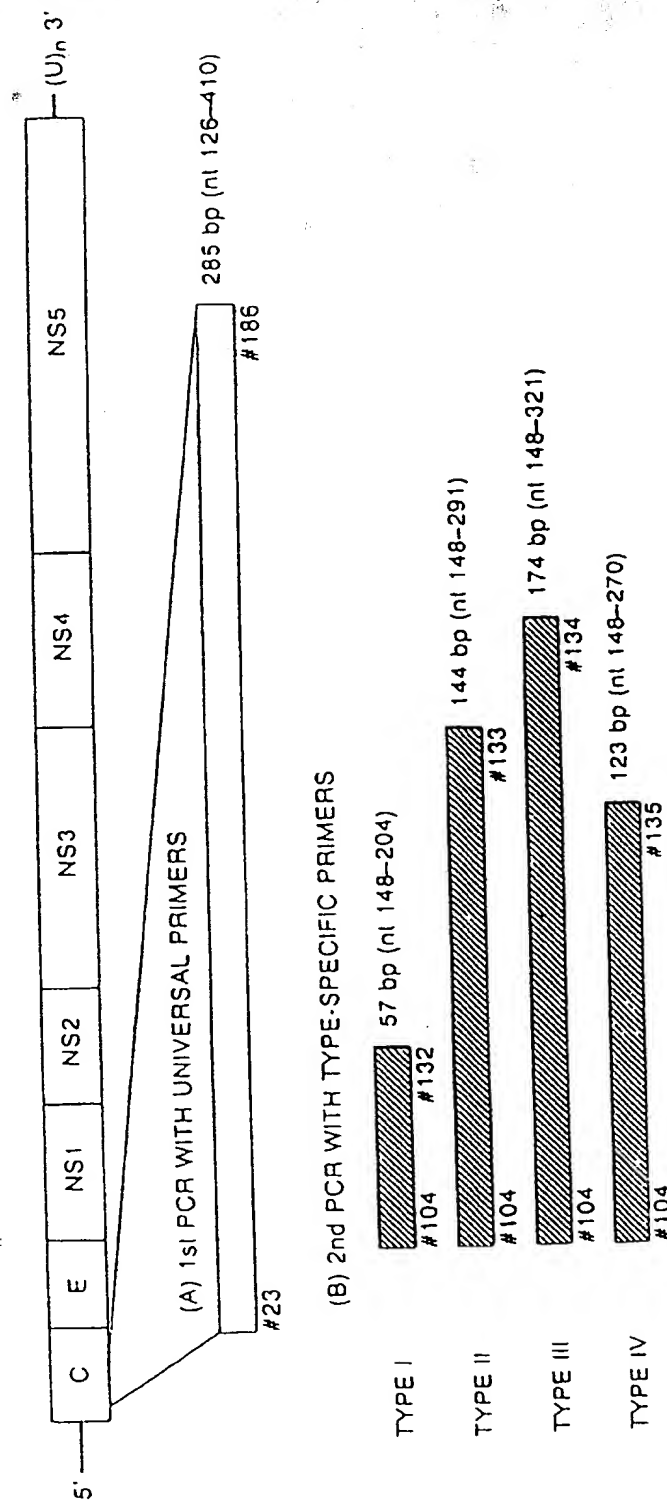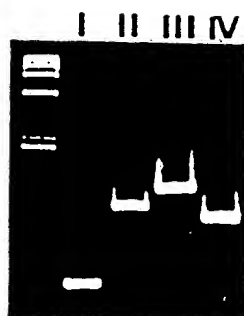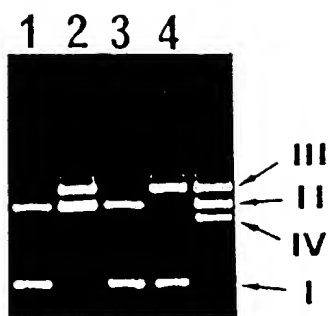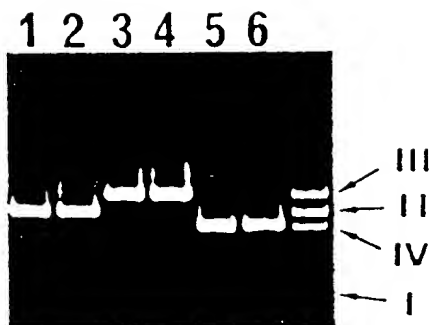
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Fig. 7

Fig. 8